

**UNITED STATES PATENT AND TRADEMARK OFFICE**  
**(Attorney Docket No. MBHB 07-1048-WO-US)**

IN RE APPLICATION OF:	)	
Sam Philip Heywood, et al	)	
	)	Examiner: David Blanchard
Serial No. 10/562,769	)	
	)	
Filed: June 27, 2006	)	Group Art Unit: 1643
	)	
Title: Modified Antibody Fragments	)	Confirmation No.: 7843

**DECLARATION OF DAVID P. HUMPHREYS**

I, David P. Humphreys, in support of the above-identified patent application, do aver and state as follows:

1. I am a co-inventor of this patent application. I also am the inventor of WO 99/15549 and U.S. 6,642,356, which have been cited against this application.
2. I received a First Class honours degree in Molecular Biology and Biochemistry from the University of Durham (UK) in 1992. I received a PhD. in from the University of Birmingham (UK) in 1995. My thesis was entitled "Expression of Human Protein Disulphide Isomerase (PDI) in *E. coli*, and protein folding *in vivo*;" this work resulted in two publications in peer reviewed journals. My PhD. research work was sponsored by the predecessor-in-interest of the assignee herein, and I have continued to work for the assignee since that time. For the last eighteen years, I have investigated issues relating to cysteine engineering and disulfide bond architecture in proteins of industrial interest, advancing from post-doctoral researcher to my present position of Director, Antibody Biology. A list of my

published papers, patent applications, and presentations is attached as Exhibit A hereto.

3. The present application arose from our search for a more efficient way of providing two or more effector molecules such as PEG on a Fab or Fab' fragment. At the time of the present invention, it was known that PEGylation of antibodies and antibody fragments could improve *in vivo* stability. It also was known that site-selective attachment of effector molecules could be achieved by attaching the effector molecules to cysteine residues in the fragment. It was known that in order to attach an effector molecule, the cysteine would have to be reduced to a thiol by subjecting the molecule to a reduction step. At the time of the invention, it was common wisdom in the art that the interchain disulfide bond between the heavy and light chains had to remain intact during this reduction step in order to preserve the antigen-binding affinity of the fragment. It was believed that if the interchain bond were absent, then the light chain might disassociate from the heavy chain, particularly once an effector molecule was attached or at the high protein concentrations commonly used for final formulation of antibody therapeutics. Therefore, reduction reactions were carried out under mild conditions, so that the interchain bond cysteines would not be reduced. These mild reactions conditions resulted in inefficient PEGylation reactions.
4. Prior to the present invention, mutations to create Fab' antibody fragments lacking inter-chain (LC-HC) bonds were known, but these Fab' fragments had not been PEGylated. Specifically, Rodrigues et al (1993) and WO 99/15549 taught that Fab' lacking inter-chain disulphide bonds could be expressed and purified using normal methods. Both of these references are directed to improving the efficiency of F(ab')<sub>2</sub> formation *in vitro* from Fab' fragments. Neither Rodriguez et al. (1993) nor WO 99/15549 taught or suggested PEGylation of those Fab' fragments, although WO 99/15549 taught PEGylation of certain species of di-Fab'. None of these works, or to my

knowledge any other prior art, taught or suggested that Fab' lacking inter-chain disulphide bonds might be suitable for *in vivo* applications.

5. Other references from that time period demonstrate the then-prevailing belief in the importance of maintaining the interchain disulphide bond. At the time of our work a wide spectrum of diseases associated with cancers of the blood were known to involve the production and deposition on and in organs of patients of 'abnormal immunoglobulins.' These include amongst others 'heavy chain deposition disease' and 'light chain deposition disease.' One of the well known manifestations of such diseases are the presence of 'Bence-Jones' proteins, which are light chain dimers that can be detected in the urine (Bradwell et al., The Lancet (2003) 361: 489-491). Bence Jones proteins appear to have multiple origins including over-expression of LC, LC variable region mutations and truncations in antibody heavy chain genes, typically C<sub>H</sub>1, C<sub>H</sub>1-hinge or even C<sub>H</sub>1-hinge-C<sub>H</sub>2 (Cogné et al, J. American Soc. Hematology (1992) 79:2181-2195). In one specific example, the human myeloma IgG1 'Dob' was shown to have a 15 amino acid deletion of the hinge sequence EPKSCDKTHTCPPCP (Steiner and Lopes Biochemistry (1979) 18:4054-4067). Hence 'Dob' was known to lack the C<sub>H</sub>1 cysteine involved in the LC-HC disulfide bond and was shown to form an aberrant LC-LC disulfide bond. In light of this combined knowledge, we were concerned that Fab-PEG or Fab'-PEG with long serum half-life, lacking an interchain LC-HC disulfide bond might be capable of LC loss or LC exchange.
6. Humphreys et al., (1998), of which I am the principle author, showed that increased stability of inter-hinge linkages through increased numbers of disulphide bonds resulted in increased serum permanence of F(ab')<sub>2</sub> molecules in rats. This supported the prevailing belief that the natural inter-chain (LC-HC) disulphide might indeed be required for full stability of molecules with longer circulation times, thus teaching one skilled in the art away from the use of fragments having no inter-chain covalent bonds. Hong

and Nisonoff (1965) reported that breakage of inter-chain (LC-HC) bonds resulted in at least some dissociation of light chain from heavy chain of a rabbit antibody, as observed during chromatographic purification. This suggested that the absence of an inter-chain covalent bond presented at least some risk of LC loss or exchange in circulating serum. Hence at the time of the present invention we were uncertain as to how robust Fab or Fab' fragments with multiple effector molecules and lacking inter-chain (LC-HC) bonds would be during circulation in serum over 1-2 weeks.

7. Fab' fragments that had been site-specifically mono-PEGylated at the hinge were known, but only those with intact inter-chain (LC-HC) bonds (cf. Chapman et al. (1999)). This is consistent with the general understanding in the art at that time that the inter-chain bond had to be intact in order to maintain good binding affinity to antigen. This reference is also consistent with the then-prevailing view that effector molecules were most desirably attached at the hinge portion of the fragment in order to prevent interference with the antigen binding function of the fragment.
8. The present invention relates to Fab' and Fab antibody fragments lacking inter-chain (LC-HC) bonds and having two or more effector molecules, at least one of the effector molecules being attached at either the light chain or the heavy chain of the fragment. At the time of the present invention, little was known about how to predict the stability *in vivo* of Fab and Fab' molecules with altered structures. It was known that 'unstable' molecules such as scFv and human IgG4 were prone to 'domain exchange' both *in vitro* and *in vivo*. Since the long serum permanence was conveyed by the PEG molecule which was covalently attached to the heavy chain, it seemed highly plausible that in Fab'-PEG lacking inter-chain (LC-HC) bonds the light chain might be exchanged or lost in the circulation. This would have resulted in loss of antigen binding function (loss of efficacy) or increased clearance of the protein through precipitation, aggregation or proteolysis.

9. The middle hinge of human IgG4 differs from that of human IgG1 in both sequence (CPSC vs CPPC) and in ability to form a ‘half-molecule.’ It was known at the time that IgG4 was able to ‘domain exchange’ in plasma to form monovalent, bispecific molecules, Schuurman et al., 1999. It had been proposed that this domain exchange was enabled by the ‘unstable’ IgG4 hinge sequence (Aalberse et al. 1999), mutation of which to CPPC was known to effectively stop the formation of the closely linked ‘half-molecule’ *in vitro* (Angal et al., 1993). Hence at the time of our experiment it was known that ineffective interchain disulphide stabilisation could result in efficient exchange between non-covalent protein:protein interfaces in Fc domains encoded by CH2-CH3. It seemed highly plausible that Fab’ light chain could be lost from our molecule in circulating serum.
10. In our experiments, we modified either the light chain or the heavy chain of the Fab’ fragments by replacing one of the cysteines that had formed the interchain disulfide bond with another amino acid, thereby destroying the interchain bond, and ensuring that the site of cysteine replacement could not subsequently become a site for attachment of an effector molecule. As preservation of the interchain disulfide bond was no longer a criterion for the reaction, we could use stronger reaction conditions during the reduction of the cysteines to thiols, allowing for more efficient multiple PEGylation; in fact, as reported in Example 1 of the specification, we were able to achieve >65% multiple PEGylation with either two or three PEG molecules using TCEP reductant. Typically di- or triPEGylation was achieved at between 75 and 85% efficiency as shown in Figure 2 of the application. Surprisingly, our modified Fab’ antibody fragments with 2 or 3 PEG molecules and having no covalent bond between the light and heavy chains were active and stable in the circulation of mice for over 140 hours. Further, there was no loss of antigen binding affinity. This result was wholly unexpected, because the literature at the time suggested that the absence of an interchain bond would result in

instability of the fragment in terms of loss or exchange of the light chain *in vivo* or proteolytic degradation. We had believed that this instability could be even greater if a large PEG effector molecule was bound to the light or heavy chain. This stability of Fab and Fab' PEGylated in this way was especially unexpected since hydrated PEG molecules were known to have an effective molecular size far in excess of 20kDa, typically in excess of 1 megaDa (Koumenis et al., Int. J. Pharmaceutics (2000) 198:83-95). Hence the potential for disruption or distortion of protein structure was perhaps significantly enhanced by the attachment of multiple large PEG molecules to proteins lacking interchain disulfide bonds.

11. I have read the references cited against the present application. Chapman et al. (Nature Biotechnology, 17:780-783, 1999), and discussed above, discloses that Fab' fragments having intact inter-chain disulfide bonds and having a single PEG attached at the hinge region have improved half-lives without loss of antigen-binding affinity. Fig. 2 of the Chapman et al. disclosure specifically shows an intact covalent bond between the light and heavy chains, thus teaching away from the presently claimed invention. To me as one skilled in the art, this reference suggests that in order to have improved stability *in vivo*, an interchain bond is necessary, and all effector molecules must be attached to the hinge. Chapman does not teach or suggest to one skilled in the art that any modification should be made to either the light chain or the heavy chain of the Fab' fragment, or that attachment of an effector molecule should occur anywhere other than the hinge.
12. My own patent publication WO 99/15549 and its equivalent U.S.6,642,356 are directed to the formation of dimeric Fab's (i.e., F(ab')<sub>2</sub>) in E. coli., because in clinical applications it is often desirable to have the increased effective binding affinity afforded by a dimeric Fab'. This patent discloses that Fab' lacking inter-chain disulphide bonds could be expressed and purified using normal methods. Example 1 of this patent describes the production of

di-Fab' from Fab' in E. coli. As stated in the patent (col. 9, lines 59-67), to minimize any possible incorrect interchain disulphide bonds between hinge regions and any other cysteines the interchain disulphide bond was removed from all Fab' constructs, by changing the interchain cysteines of cKappa and C<sub>H</sub>1 to serines. In particular, both cysteines were removed to minimize such incorrect bonds when the two Fab' fragments come together to form the di(Fab')<sub>2</sub> dimer. The issue of such incorrect bonds does not arise when the fragments are to be used in the form of un-dimerized Fab'. At no time in this example, or in this patent, is PEG or any other effector molecule attached to a Fab' fragment. The patents reports the discovery that di-Fab' formation *in vivo* in the periplasm of E.coli is an inefficient process that is modulated, *inter alia*, by hinge sequence and complexity (col. 14, lines 33-35). It was found that Fab' fragments with unmodified  $\gamma$ 1 hinges gave the greatest F(ab')<sub>2</sub> yield *in vivo*, which would suggest to one skilled in the art that unmodified hinge regions were preferred.

13. This patent discloses in Example 2 that a di-Fab' with an intact interchain disulfide and a modified hinge with 4 cysteine residues was PEGylated with an efficiency of  $\leq 1.3\%$ . This PEGylation was reasoned to be hinge specific. Firstly since there were more cysteines in the dimerised hinge (8) than in interchain disulfide cysteines (4) there was a greater chance of reduction and PEGylation of the hinge. Secondly, the inter-hinge disulphide bonding was known to be somewhat heterogeneous meaning that there were likely to be some 'free' cysteines available for PEGylation. Thirdly, the reducing conditions used (1.3mM  $\beta$ -ME, pH6.0, 37°C for 45 minutes) were so mild as to be unable to effectively reduce interchain (LC-HC) bonds. Prior art (King et al., Cancer Research 1994 54:6176-6185) had shown that more strongly reducing conditions (5mM  $\beta$ -ME, pH6.0, 37°C for 30 minutes) were unable to reduce interchain (LC-HC) bonds (see Figure 3).

14. The SEQ ID NO:1 in the '356 patent is  $N$ TCPPCPXYCPPCPA<sup>C</sup>, wherein X and Y which may be the same or different are each a neutral aliphatic L-amino acid residue. In one useful peptide X is an alanine residue and Y is a threonine residue. The SEQ ID NO:1 in the present application is DKTHTCPP whilst the SEQ ID NO:2 is DKTHTCAA. SEQ ID NO:1 and SEQ ID NO:2 of the '356 patent are not the same as SEQ ID NO:1 or SEQ ID NO:2 of the present application. In particular the sequences for the hinge region in the '356 patent contain 4 cysteine groups. The hinge regions in the antibody fragments of the present application contain one or two cysteine groups. The additional cysteines in the hinge regions of the '356 patent fragments allow the Fab' fragments to dimerize with one another. In the present application the goal is not to dimerize the Fab' fragments. The sequence of the '356 patent could not be used in the Fab' fragments of the present application.
15. The Hsei reference suggests PEGylation of various antibody fragments, but does not teach any Fab or Fab' fragment having more than one polymer molecule attached wherein one of the molecules is attached at a site other than the hinge. Hsei teaches that a polymer molecule can be coupled to the light or heavy chain of a Fab or Fab' only when there is only one polymer molecule attached to the fragment. Hsei teaches nothing about modification of the interchain disulfide bond when the PEGylation takes place at the hinge. Hsei teaches that one of the interchain cysteines is to be replaced with a serine only when there is only one effector molecule attached to the fragment. Hsei teaches nothing about modification of the interchain disulfide bond when the PEGylation takes place at the hinge.
16. Hsei describes but does not exemplify site specific attachment of a PEG molecule to any cysteine other than a single cysteine in the hinge. Hsei



describes but does not exemplify attachment of two PEG molecules to Fab or Fab'. Hsei describes and exemplifies attachment of 2 PEG molecules to F(ab')<sub>2</sub> molecules, but only by using random amine (lysine) specific NHS-PEG chemistries followed by chromatographic separation of unPEGylated, monoPEGylated and diPEGylated F(ab')<sub>2</sub> fragments. Said F(ab')<sub>2</sub> molecules were formed from Fab' molecules with a C-terminal leucine zipper dimerisation motif followed by proteolytic cleavage and removal of the leucine zipper. Hsei neither describes nor exemplified methods for the reduction / activation of interchain cysteines for PEGylation without disruption of the inter-hinge disulphide bonds which stabilize the dimeric F(ab')<sub>2</sub> structure. Such a 'random PEGylation' approach was shown to be inefficient and wasteful by Hsei and had been evidenced in the prior art, (e.g. Pedley et al., Br. J. Cancer 1994 70:1126-1130, and Delgado et al., Br. J. Cancer 73:175-182).

17. The approach taken by Hsei is clearly differentiated from the approach taken in the present application which enables specific control over both the site and number of PEG molecules attached, namely, attachment of two or more PEG molecules to a Fab or Fab' fragment, and wherein at least one of the PEG molecules is attached to a cysteine in the heavy or light chain constant region. In addition our application describes precise methods for very efficient PEGylation reactions. Since Hsei did not make Fab' variants containing disrupted interchain disulphide bonds it was also not possible to foresee that one such variant forms very effectively an interchain disulphide bond between the C-terminal cKappa cysteine and the single hinge cysteine (see lane 4 figure 3b in our application), nor then that such a disulphide bond would retain very significant Fab' thermal stability (see pDPH225 in Table 1 of our application). Hsei does not teach any practicable or efficient method of PEGylating such molecules. The present application shows the surprising result that even 5mM DTT compared to the 0.2mM DTT described in Hsei is ineffective at reducing any such 'unexpected' disulphide bonds to the extent

required for high levels of PEGylation (see Figure 1 in our application). Furthermore, we show in our application that stronger reducing conditions using different classes of reducing reagents (such as TCEP) are required in order to result in efficient PEGylation of such disulphide engineered Fab and Fab' molecules. It is also clear that 'strong' reducing reagents such as TCEP could not be used to reduce and PEGylate F(ab')<sub>2</sub> molecules without reducing them to Fab'. As one skilled in the art, this reference does not teach or suggest to me that it would be desirable to attach two or more effector molecules to such a Fab or Fab' fragment, and provides no motivation or methods for doing so.

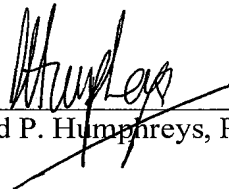
18. In my opinion as one skilled in the art of antibodies, one skilled in the art would not combine the Chapman, Hsei, and Humphreys references in the manner suggested by the patent examiner. Specifically these references would not teach one skilled in the art to place effector molecules on both the hinge and the light or heavy chain at the site of the interchain cysteine bond, as suggested by the Examiner. There is no reason for one skilled in the art to combine these references. Moreover, the prevailing view in the art that destroying the interchain bond would diminish antigen binding affinity would lead one skilled in the art away from removal of both interchain cysteines.
19. One aspect of the present invention is an intermediate in which the interchain cysteine of C<sub>H</sub>1 of a Fab' fragment has been replaced by another amino acid, and wherein the C<sub>L</sub> cysteine is covalently bonded to a cysteine in the hinge region. This fragment served as an intermediate that could be PEGylated to provide the PEGylated fragments of the present invention. The formation of this bond was unexpected by me and by my co-inventor. We also were surprised to find that this intermediate fragment provided greater stability than when both interchain cysteines were mutated, as shown at page Table 1 of the present application (compare pDPH224 and pDPH225). This greater thermal stability enabled precipitation and removal of many E.coli host proteins and Fab' proteolytic fragments by incubation of E.coli periplasmic extract at

elevated temperature. Because the light chain cysteine and hinge cysteine were bound to each other and not available for extraneous bonding, the intermediate fragment also provided greater analytical simplicity, purity, and protein integrity.

20. I have reviewed reference WO 93/06217 to Carter, which relates to expression in E.coli of antibody fragments having at least a cysteine present as a free thiol, used for the production of bifunctional F(ab')<sub>2</sub> antibodies. In particular, Carter desires to provide Fab' antibody fragments having at least one hinge region cysteine present as a free thiol (Fab'-SH) while obviating the inherent problems in generating Fab'-SH from intact antibodies (p. 5, lines 24-31). Thus Carter discloses a Fv polypeptide containing an immunoglobulin heavy chain Fv region and an immunoglobulin light chain Fv region, said light or heavy chain also comprising an unpaired cysteinyl residue as a free thiol, and recovering the polypeptide under conditions that substantially maintain the cysteinyl residue as the free thiol. (page 6, lines 9-14) Carter also discloses a Fab' antibody polypeptide having at least one hinge region cysteine present as a free thiol (Fab-SH) (p. 7, lines 4-18).
21. As one skilled in the art, it is apparent to me that the fragments as taught by Carter do not inherently form bonds between the cysteine on the light chain and the hinge cysteine. Because in Carter the cysteine is present as a free thiol and maintained as a free thiol until it can be reacted to form F(ab')<sub>2</sub> dimers (page 8, lines 6-35), the hinge cysteine thiol was not available to react with a light chain cysteine thiol. Moreover, since Carter was not interested in adding effector molecules to the fragment, Carter would not have realized that a Fab' fragment with a bond between the light chain cysteine and the hinge cysteine would have conferred any advantage in a subsequent PEGylation step.

22. I hereby state that I have been warned that willful false statements and the like are punishable by fine or imprisonment, or both (18 U.S.C. 1001), and that such willful false statements may jeopardize the validity of the application or document or any registration resulting therefrom, and I declare that all statements made of my own knowledge are true; and all statements made on information and belief are believed to be true.

Date: 17<sup>th</sup> August 2010

  
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David P. Humphreys, PhD.

## **Publications**

**Humphreys DP** and Bowering L (2009) Production of antibody Fab' fragments in *E. coli*, in Therapeutic Monoclonal Antibodies - from bench to clinic. Wiley and sons. p589-622.

Heywood SP and **Humphreys DP** (2009) Polymer fusions to increase antibody half-lives: PEGylation and other modifications, in Recombinant Antibodies for Immunotherapy. Cambridge University Press p275-292.

**Humphreys D P, et al.,** (2007). Alternative antibody Fab' fragment PEGylation strategies: combination of strong reducing agents, disruption of the interchain disulphide bond and disulphide engineering. *PEDS* **20**: 227-234.

**Humphreys D P** (2007). Periplasmic expression of antibody fragments, in *The Periplasm*. ASM Press, Washington DC, pp361-388.

**Humphreys D P** (2004). Engineering of *Escherichia coli* to improve the purification of periplasmic Fab' fragments: changing the pI of the chromosomally encoded PhoS/PstS protein. *PROT. EXPR. PURIFIC.* **37**: 109-118.

Glover, D.J., and **Humphreys D P** (2004). Antibody fragments: production, purification and formatting for therapeutic applications, in *Antibodies Volume 1: production and purification*. Kluwer academic, London, pp. 25-73.

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**Humphreys D P, and Glover D J** (2001). Therapeutic antibody production technologies: molecules, applications, expression and purification. *CURR. OPIN. DRUG DISC. DEV.* **4**: 172-185.

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**Humphreys D P, et al.** (2000). Improved efficiency of site-specific copper(II) ion-catalysed protein cleavage effected by mutagenesis of cleavage site. *PROTEIN ENG.* **3**: 201-206.

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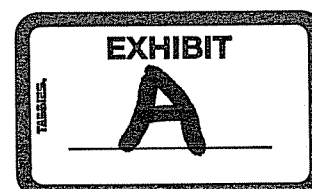
**Humphreys D P, et al.** (1996). Co-expression of human protein disulphide isomerase (PDI) can increase the yield of an antibody Fab' fragment expressed in *E. coli*. *FEBS LETTERS* **380**: 194-197.

**Humphreys D P, et al.** (1995). Human protein disulfide isomerase functionally complements a *dsbA* mutation and enhances the yield of pectate lyase C in *E. coli*. *J. BIOL. CHEM.* **270**: 28210-28215.

Stafford S J, **Humphreys D P**, and Lund P A (1999). Mutations in *dsbA* and *dsbB*, but not *dsbC*, lead to an enhanced sensitivity of *E. coli* to Hg<sup>2+</sup> and Cd<sup>2+</sup>. *FEMS LETTERS.* **174**: 179-184.

## **Presentations: Oral**

- "The cell as a manufacturing site" TOPRA Biotechnology, London 13<sup>th</sup> March 2008.
- "What is the potential for antibody fragments ?" BioProduction, Berlin 30<sup>th</sup> October 2007.
- "Bacterial and yeast expression systems" TOPRA Biotechnology April 2007.



- "Antibody Fab' fragments: expression and purification from *E. coli*, PEGylation and clinical uses." Keystone, Lake Louise, 3<sup>rd</sup> Feb 2007.
- "Multi-PEGylation, an alternative and high efficiency strategy for the PEGylation of therapeutic antibody Fab fragments." HAH, Montego Bay, 12<sup>th</sup> May 2006.
- "PEGylation of antibody Fab' fragments: expression and purification from *E. coli*, PEGylation and clinical uses." World Biopharm Forum, Queens college Cambridge UK, 18<sup>th</sup> April 2006.
- "PEGylation of antibody Fab' fragments produced in *E. coli*." IBC Drug Disc. Tech., Hammersmith, London, 14<sup>th</sup> March 2006.
- "Production of antibodies and antibody fragments in microbial systems." Modern Drug Discovery & Development, San Diego, 19th October 2004.
- "Engineering of *E. coli* to improve the purification of periplasmic Fab' fragments: changing the pI of the chromosomally encoded PhoS/PstS protein". HAH, Dublin 8<sup>th</sup> October 2004.
- "CASE study: Simplifying the purification of periplasmic Fab', the creation an use of new *E. coli* strains". Visiongain Strategies for monoclonal antibody therapy 2004, London Kensington Hilton, 1-2 July 2004.
- "Engineering of *Escherichia coli* to improve the purification of periplasmic Fab' fragments: changing the pI of the chromosomally encoded PhoS / PstS protein". CHI Recombinant Antibodies conference, Cambridge MA, 28<sup>th</sup> April 2004.

### **Posters**

"Multi-PEGylation, a new strategy for the high efficiency PEGylation of therapeutic antibody Fab' fragments" IBC Antibody Engineering, San Diego, 3-8<sup>th</sup> December 2005.

"Engineering of *Escherichia coli* to improve the purification of periplasmic Fab' fragments: changing the pI of the chromosomally encoded PhoS / PstS protein". IBC Antibody Engineering, San Diego 1-4 December 2003.

"High yielding and cost effective production of humanised Fab' fragments in *E. coli*". IBC Antibody Engineering, San Diego 3-6 December 2000.

"High yielding and cost effective production of functionally different humanised antibody fragments in *E. coli*". Medicon Valley Association, Malmö, Sweden 20-21 September 2000.

"High yielding and cost effective production of functionally different Humanised Antibody fragments in *E. coli*". Human Antibodies and Hybridomas, Edinburgh, 8-10 Sept. 1999.

"F(ab')<sub>2</sub> molecules with hinge sequences conferring increased resistance to chemical reduction *in vitro* and increased serum permanence times in an animal model". Therapeutic Antibodies, San Francisco 21-24 September 1997

"Co-expression of Human Protein Disulphide Isomerase (PDI) can increase the yield of an antibody Fab' fragment expressed in *Escherichia coli*". NIH conference on Protein Folding, Washington DC, 23-26 April 1996.

### **Patent applications**

WO2005/003169 Fab PEGylation construct.  
 WO2005/003170 MultiPEGylation.  
 WO2005/003171 Disulphide engineering / PEGylation.  
 WO2004/035792A1: PhoS strain engineering.  
 WO2003/004636A2: Bacteriophage signal peptides.  
 WO0032795A1: Copper cleavage peptides.  
 WO9915549A2 / US6642356B1 / EP1015495B1: Hinge sequences.  
 GB 95/18383.6, "Microbiological process".

